doi:10.1093/brain/awq086 Brain 2010: 133; 1300–1311 | 1300

BRAIN
A JOURNAL OF NEUROLOGY

REVIEW ARTICLE

Narcolepsy: autoimmunity, effector T cell activation due to infection, or T cell independent, major histocompatibility complex class II induced neuronal loss?

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Human narcolepsy with cataplexy is a neurological disorder, which develops due to a deficiency in hypocretin producing neurons in the hypothalamus. There is a strong association with human leucocyte antigens HLA-DR2 and HLA-DQB1*0602. The disease typically starts in adolescence. Recent developments in narcolepsy research support the hypothesis of narcolepsy being an immune-mediated disease. Narcolepsy is associated with polymorphisms of the genes encoding T cell receptor alpha chain, tumour necrosis factor alpha and tumour necrosis factor receptor II. Moreover the rate of streptococcal infection is increased at onset of narcolepsy. The hallmarks of anti-self reactions in the tissue—namely upregulation of major histocompatibility antigens and lymphocyte infiltrates—are missing in the hypothalamus. These findings are questionable because they were obtained by analyses performed many years after onset of disease. In some patients with narcolepsy autoantibodies to Tribbles homolog 2, which is expressed by hypocretin neurons, have been detected recently. Immune-mediated destruction of hypocretin producing neurons may be mediated by microglia/macrophages that become activated either by autoantigen specific $CD4^+$ T cells or superantigen stimulated $CD8^+$ T cells, or independent of T cells by activation of DQB1*0602 signalling. Activation of microglia and macrophages may lead to the release of neurotoxic molecules such as quinolinic acid, which has been shown to cause selective destruction of hypocretin neurons in the hypothalamus.

Keywords: molecular mimicry; T cell receptor; sleep; cytokines

Abbreviations: HLA = human leucocyte antigen; MHC = major histocompatibility complex; TCR = T cell receptor; TNF = tumour necrosis factor; Trib2 = Tribbles homolog 2

Received November 30, 2009. Revised and Accepted March 23, 2010. Advance Access publication February 19, 2010

Introduction

Narcolepsy with cataplexy—a sudden, short loss of muscle tone triggered by emotions—is a disabling chronic brain disorder characterized by excessive daytime sleepiness, sleep paralysis, hallucinations and disturbed nocturnal sleep. Although the severity of daytime sleepiness is fluctuating, it is present most of the time. Daytime sleepiness ranges from mild sleepiness that is easily overcome to excessive overwhelming and irresistible daytime sleepiness. The latter may manifest itself by episodes of daytime sleep occurring without warning ('sleep attacks') (Dement *et al.*, 1976; Bassetti and Aldrich, 1996). The prevalence of narcolepsy with cataplexy falls between 25 and 50 per 100 000 people (Longstreth *et al.*, 2007). There seems to be a slight male predominance. Age at onset is between 15 and 40 years in most cases.

Up to 95% of patients with narcolepsy and cataplexy have low CSF hypocretin-1 levels (Nishino *et al.*, 2000; Baumann and Bassetti, 2005; Bourgin *et al.*, 2008). Autoptic data suggest that this deficiency reflects a loss of hypothalamic neurons which produce hypocretin peptides (hypocretin-1 and hypocretin-2; also known as orexins A and B) (Thannickal *et al.*, 2000), which in turn bind to hypocretin receptors (hypocretin receptor-1 and hypocretin receptor-2). The hypocretin system in sleep, wakefulness and narcolepsy has been discussed in detail elsewhere (for review see Sakurai, 2007). In brief, two independent studies in 1999 showed that mutations in the hypocretin-2 receptor gene are responsible for canine narcolepsy-cataplexy and a gene deletion of hypocretin in mice leads to a phenotype strikingly similar to human narcolepsy (Chemelli *et al.*, 1999; Lin *et al.*, 1999). Hypocretin-1 and hypocretin-2, which are derived from a common precursor peptide, the prepro-orexin, share significant homology in their C-terminal part. Hypocretin-1 binds to two G protein-coupled receptors named hypocretin receptor-1 and -2. Whereas the latter is a non-selective receptor for both peptides, hypocretin receptor-1 is selective for hypocretin-1. Both hypocretin-1 and -2 are exclusively produced in the lateral hypothalamic area; their respective receptors are expressed in the entire CNS. In regard to the narcolepsy-like phenotype in animals with non-functional hypocretin or hypocretin receptor genes it is remarkable that hypocretin-1 and -2 increase wake time and decreased rapid eye movement and non-rapid eye movement sleep time. The inability to maintain wakefulness seems to depend critically upon hypocretin-2, while the profound dysregulation of rapid eye movement sleep control emerges from loss of signalling through both hypocretin receptor-1 and hypocretin receptor-2-dependent pathways (Willie *et al.*, 2003); for review see Ohno and Sakurai (2008).

In humans, only one patient with an early onset of disease in childhood has been reported to have a mutation in the hypocretin gene (Peyron *et al.*, 2000). The aetiology of the reduction in the number of neurons containing detectable pro-hypocretin mRNA or hypocretin-like immunoreactivity in the hypothalamus in narcolepsy remains unexplained. Low hypocretin concentrations in the CNS may point to a failure of the neurons to produce hypocretin. In the normal hypothalamus, 80% of the hypocretin-producing neurons also express prodynorphin and neuronal activity-regulated pentraxin. The number of neurons expressing these gene products is reduced in proportion to the loss of hypocretin neurons (Blouin *et al.*, 2005; Crocker *et al.*, 2005). The selectivity of the loss of hypocretin neurons in the hypothalamus is shown by the absence of reduction in the number of neurons expressing melanin-concentrating hormone (Thannickal *et al.*, 2009). In this review we will focus on immunological mechanisms possibly involved in the pathophysiology of the disease.

Why should narcolepsy have anything to do with immunology? It is the association with *HLA-DR2* genes and a newly discovered T cell receptor alpha polymorphism

Narcolepsy is genetically characterized by strong linkage to distinct human leucocyte antigen (HLA) alleles. A genetic association of narcolepsy with HLA-DR2 and HLA-DQ1 in the major histocompatibility (MHC) region was described more than 20 years ago (Langdon *et al.*, 1984). High resolution typing by DNA techniques has further characterized the DR2 and DQ1 serological specificities associated with narcolepsy. As reviewed recently, the association between HLA class II genes and narcolepsy was present in all ethnic groups and the most tightly linked HLA allele was DQB1*0602 (Tafti, 2009). Whereas this allele is present in only 12–38% of the general population, more than 85% of the patients with narcolepsy-cataplexy have the HLA DQB1*0602 allele, most often in combination with HLA-DR2 (DRB1*1501) (Mignot, 1998). Moreover, the DQB1*0602 allele alone, particularly when homozygous, was the major narcolepsy susceptibility allele in different ethnic groups including African-Americans, Caucasian-Americans and Japanese (Mignot *et al.*, 2001). Several alleles have been identified that appeared to be protective (DQB1*0601, DQB1*0501 and DQA1*01) (Mignot *et al.*, 2001).

Among genetic factors linked to autoimmune disease development, MHC class II (MHCII) genes on chromosome 6 account for the majority of cases of familial clustering in common autoimmune diseases, and have also been linked to sporadic forms. In systemic lupus erythematosus, the most consistent HLA associations are with the MHCII allotypes, HLA-DR3 and HLA-DR2. A pre-eminent role of the extended haplotype defined by HLA-DRB1*1501 has also been highlighted in recent studies on multiple sclerosis (Fernando *et al.*, 2008). The mechanisms that account for MHCII associated anti-self immunity remain poorly defined. Tolerance to self-antigens is achieved by deletion of T cell precursors that express T cell receptors (TCRs) having high avidity for self-antigen—MHC complexes expressed on dendritic cells and epithelial cells in the thymus. Peripheral immune tolerance mechanisms control mature T cells that bear a TCR of low avidity for self-antigen—MHC complexes and that escape from the thymus to the periphery (Mueller, 2010). A commonly held view is that disease-associated HLA allotypes promote a breach of peripheral self-tolerance because they favour the presentation of specific self-peptides to autoreactive T cells. Alternatively, the disease-associated HLA allotypes could bias the TCR repertoire generated during T cell development in the thymus towards the selection of potentially pathogenic autoreactive specificities. It has also been proposed that autoimmunity might be promoted by ectopic or inappropriately high levels of HLA expression in the diseased tissues. An intriguing observation has been provided by crystallographic studies using a soluble form of DQ0602 complexed with a peptide from human hypocretin (amino acids 1–13) (Siebold *et al.*, 2004). The hypocretin peptide is presented in the DQ0602-binding grove with peptide side chains anchored in the P4 and P9 pockets. These pockets differ significantly between the DQ0602 narcolepsy susceptibility molecule and DQ0601, an allele that is protective. Since no anti-self immunity to hypocretin has been detected so far, the significance of these studies remains open.

The hallmarks of T cell involvement in autoimmune diseases are the presence of T cells sensitized to self-antigens, dysregulated effector $CD4^+$ T cells, such as T helper 17 cells, low titres of regulatory T cells, and inflammation at the sites of autoimmune attack. The inflammatory reaction is typically characterized by a local accumulation of $CD4^+$ T cells and proinflammatory macrophages with increased expression of MHCII and cytokines. None of these characteristic features of T cell autoimmunity have been documented in narcolepsy. However, in a highly interesting recent study on T cell receptor alpha (TCRα) or -beta (TCRβ) subtypes, 807 narcolepsy patients positive for HLA-DQB1*0602 and exhibiting hypocretin deficiency in the CSF, as well as 1074 controls were selected for a genome-wide association study. The data identified an association between narcolepsy and polymorphisms in the TCRα locus. The TCRα chain is part of the TCR of $CD8^+$ T cells, which recognize antigens presented by HLA class I molecules, and $CD4^+$ T cells, which recognize antigens presented by HLA class II molecules, including the DQα (alpha) β (beta) heterodimer denoted DQ 0602, which is encoded by the DQB1*0602 and DQA1*0102 alleles. Somatic recombination in the TCRα and TCRβ loci in developing T cells leads to the generation of a diverse repertoire of distinct TCRα β idiotype-bearing T cells. Since narcolepsy is almost exclusively associated with a single HLA allele—DQB1*0602—the authors suggest that the TCRα polymorphism could contribute to autoimmunity directed against hypocretin neurons by influencing the occurrence of variable-joining region VJ2 recombinations that can interact with DQ0602 (Hallmayer *et al.*, 2009).

Another interesting observation has recently been described in a series of experiments reported by Carla Shatz (2009). Since the initial report of MHCI expression and activity regulation in neurons, it has been suggested that altered MHCI expression contributes to synaptic changes and learning defects (for review see Shatz, 2009). In a search for MHCI-binding receptors, TCRβ mRNA was detected in neurons. However, TCRα—the second obligatory component of a functional TCR—was not detected in neurons. As a hypothesis, polymorphism of the TCRα-β genes may influence the interaction with MHCI and thereby neuroprotection in disease states (Boulanger and Shatz, 2004). Some support for this comes from experiments with mice that lack either β2-microglobulin—a cosubunit for MHCI—or the transporter associated with antigen processing 1 (TAP1) required for loading antigen peptides onto MHCI molecules. Sciatic nerve transsection in both types of mutant mice showed axotomized α-motoneurons to have more extensive detachments of synapses than those in wild-type mice (Oliveira *et al.*, 2004). More sensitive techniques to detect TCRα and β gene expression and studies on TCR—MHCI interactions on neurons are required to come to conclusions on the significance of MHCI expression by neurons.

The strong association of narcolepsy with HLA-DQB1*0602 has prompted interest in the hypothesis that narcolepsy is an autoimmune disease. However, several issues relevant to this model deserve emphasis.

(i) From a clinical point of view, a specific autoimmune disease is often associated with various other autoimmune manifestations in the affected individual or in the family of the patient. Classical autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis or myasthenia gravis have not been reported to be increased in narcoleptic patients or their families. In fact, other autoimmune diseases (e.g. systemic lupus erythematosus, multiple sclerosis and neuromyelitis optica with anti-aquaporin-4 antibodies) have been observed only very rarely in narcoleptic patients (Younger *et al.*, 1991; Pablos *et al.*, 1993; Baba *et al.*, 2009).

(ii) Unlike the situation observed in other autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and Sjögren syndrome, associated autoantibodies such as antinuclear antibodies, antibodies to nDNA, SS-A, Sm, histone and rheumatoid factor are not increased in narcolepsy (Rubin *et al.*, 1988). It is of note that no data are provided about the duration of the disease at the time point when the sera were taken for the study.

(iii) Intrathecal synthesis of immunoglobulins and oligoclonal bands are only rarely seen in the CSF of narcoleptic patients. One study reported two out of 15 patients to have oligoclonal bands and one of these to have an increased IgG index in the CSF. These patients had narcoleptic symptoms for 7 and 33 years, respectively (Fredrikson *et al.*, 1990). In another study, four of 22 patients with narcolepsy showed oligoclonal bands in the CSF. The disease duration was 8, 10, 12 and 30 years, respectively. Measurement of antibodies to various viruses showed three patients to be positive for the herpes simplex virus (two patients) or cytomegalovirus (Schuld *et al.*, 2004). The assays used are not sensitive enough to detect the production of antibodies to CNS antigens.

(iv) There is so far no evidence for the presence of antibodies to hypocretin or hypocretin receptor in the disease and antibodies to Tribbles homolog 2 (Trib2) which is expressed by hypocretin producing neurons have been detected in only 14% of narcoleptic patients (see below).

Table 1 Autoimmunity in narcolepsy?

	References
Pro	
Association with HLA-DR2/DQB1*0602	Langdon *et al*., 1984; Matsuki *et al*., 1992
Polymorphism in the TCRα locus	Hallmayer *et al*., 2009
Dysregulation of TNF/TNF receptor system	Hohjoh *et al*., 2001*a*; Okun *et al*., 2004; Himmerich *et al*., 2006
Increased antibodies to streptolysin and to DNAseB	Billiard *et al*., 1989; Aran *et al*., 2009
Anti-Trib2 antibodies	Cvetkovic-Lopes *et al*., 2010
Contra	
Only rare association with known autoimmune diseases in patients with narcolepsy, or in their families (e.g. systemic lupus erythematosis, multiple sclerosis)	Pablos *et al*., 1993; Baba *et al*., 2009
No autoantibodies to nuclear proteins (antinuclear antibodies, anti-nDNA)	Rubin *et al*., 1988
Usually no increase in IgG index and no oligoclonal bands in CSF	Fredrickson *et al*., 1990; Schuld, 2004
No narcolepsy specific antibodies (anti-neuronal, anti-hypocretin, anti-hypocretin receptor)	Black *et al*., 2005; Tanaka *et al*., 2006
No signs of inflammation in autopsy studies and no elevation of C-reactive protein	Aran *et al*., 2009; Honda *et al*., 2009*a*; Thannickal *et al*., 2009
No TNF gene expression in the CNS	Peyron *et al*., 2000

(v) Autopsy studies have not shown an accumulation of T or B lymphocytes in the CNS, an influx of monocytes from the blood or an activation of microglia in the tissue, at least not at late time points of the disease (Table 1). The limited availability of tissues and CSF at early time points of the disease has hampered the search for autoantibodies and the detection of oligoclonal bands in CSF.

(vi) Finally, systemic markers indicating inflammation, such as increased blood sedimentation and elevated C-reactive protein cannot be demonstrated. Since intravenous immunoglobulins proved effective in the treatment of various autoimmune diseases, it is interesting to look at the response of this treatment in narcoleptic patients. Several single observations point to beneficial effects when immunoglobulins are administrated close to disease onset (Plazzi *et al.*, 2008). However, persistent improvements of narcoleptic symptoms were not observed in four other patients as recently reported (Valko *et al.*, 2008). Taken together, these clinical findings do not support a role for local or even systemic autoimmunity in narcolepsy. Several of the aforementioned issues will be discussed in more detail below.

Are anti-neuronal antibodies involved?

Loss of hypocretin neurotransmission may be due to impaired production and/or secretion of hypocretin by neurons, or result from the loss of neurons that produce hypocretin. Several studies have addressed the hypothesis that autoantibodies may lead to alterations in the hypocretin system. An increased IgG index or oligoclonal bands were detected infrequently in the CSF of patients with narcolepsy (see above). Thus intrathecal synthesis of autoantibodies by local plasma cells is not a uniform finding of the disease (Fredrikson *et al.*, 1990; Schuld *et al.*, 2004). Furthermore, recent studies have failed to detect specific antibodies against hypocretin or hypocretin receptors (Black *et al.*, 2005; Tanaka *et al.*, 2006). In these studies no data are provided in regard to the time point of disease onset and sampling of the sera. No antibodies to hypothalamic neurons became detectable in the sera of 46 narcoleptic patients, the duration of illness being 23.6 ± 10.6 years (Overeem *et al.*, 2006). Likewise, antibodies to hypothalamic neurons were documented in only one of 9 patients, and the antibody epitope was not characterized (Knudsen *et al.*, 2007). Insulin-like growth factor binding protein 3, which is expressed in hypocretin neurons and downregulated in narcolepsy, has recently been identified as a potential new autoimmune target. However, no anti-insulin-like growth factor binding protein 3 antibodies were detected in human sera or the CSF of patients. Insulin-like growth factor binding protein 3 concentrations in the CSF were not decreased (Honda *et al.*, 2009*b*). A new IgG antibody from patients with narcolepsy has been described to interfere with smooth muscle contractions in mouse colon preparations (Jackson *et al.*, 2008). The epitopes of the antibodies detected in the assay remain unclear. Hypocretin is apparently not expressed in the murine gut (Baumann *et al.*, 2008).

In a most recent, elegant search for proteins, which are expressed exclusively by hypocretin producing neurons, the screening approach came up with Trib2. While further characterization showed that Trib2 is not only expressed by hypocretin neurons, but also by other neurons, the study nevertheless points to Trib2 being an autoantigen in patients with narcolepsy (Cvetkovic-Lopes *et al.*, 2010). Using the 28 C-terminal amino acids of Trib2 in an ELISA assay, 20 (14%) of 143 narcoleptic patients had antibody titres of more than 2 SD above the mean titre of healthy controls. Only 2 (5%) out of 42 healthy controls had such antibodies (>2 SD). The anti-Trib2 antibody titres were detected more often in the first year of disease onset. Immunhistochemistry showed that antibodies recognize hypocretin neurons in the mouse hypothalamus. However, the staining pattern looks cytoplasmic, which raises the question if the autoantibody would reach its intracellular antigen—Trib2—*in vivo*. Future studies should aim at (i) developing the ELISA system further in order to find other intramolecular epitopes which harbour more dominant B cell epitopes than the C-terminal part of the Trib2 protein; (ii) testing for T cell responsiveness to Trip2 and (iii) investigating if mice immunized with Trib2 or injected with anti-Trib2 antibodies will develop a narcolepsy-like disease. Of note, three of five patients with uveitis have been identified to have anti-Trib2 antibodies (Zhang *et al.*, 2005). However, since the first description, the functional significance of the antibody detected in uveitis has not been further investigated.

Antibodies to intracellular antigens are common in old people and in a variety of infections and autoimmune diseases. However, these antibodies are not directly involved in causing disease. The same holds true for many of the anti-neuronal antibodies that characterize neurological paraneoplastic disorders. This contrasts antibodies to voltage-gated potassium or calcium channels located at nerve terminals, which may lead to limbic encephalitis and paraneoplastic cerebellar degeneration, respectively (for review see Dropcho, 2005). Limbic encephalitis may also be associated with antibodies to N-methyl-D-aspartic acid receptors (Graus *et al.*, 2008). In a recent study on 38 patients with antibodies to the onconeuronal protein Ma-2, five patients were identified with excessive daytime sleepiness, and low to undetectable hypocretin in the CSF that may indicate hypothalamic dysfunction (Dalmau *et al.*, 2004). However, anti-Ma-2 autoantibodies were not detected in patients with narcolepsy ($n = 19$), the mean duration of illness being 9.7 ± 8.3 years (Overeem *et al.*, 2004). However, the clinical spectrum of anti-neuronal antibodies may be much broader. For example, the risk of Parkinson's disease is increased among women with autoimmune diseases including Graves' disease, insulin-dependent diabetes and pernicious anaemia (Rugbjerg *et al.*, 2009). These data may indicate the presence of autoantibodies to dopaminergic neurons.

Are there signs of inflammation in the CNS in narcolepsy?

In autoimmune diseases, histological examination frequently reveals cellular infiltrates consisting of lymphocytes, plasma cells and macrophages in areas of tissue destruction. It is therefore of importance to determine whether this is seen in narcolepsy. What regions in the CNS should be analysed in depth? As outlined above, special attention should be given to hypocretin-1 and -2 producing neurons in the hypothalamus, and to the hypocretin projection fields. Since the disease does not reduce life expectancy, histological examination of brains is hardly an available option. In one patient having a *hypocretin* mutation, early onset narcolepsy (age 6 months) and a long follow-up over many years, histological analysis did not show 'obvious lesions' and the presence of a mild astrogliosis in the perifornical area remains controversial (Honda *et al.*, 2009*a*, Thannickal *et al.*, 2009). Most importantly, immunohistochemical staining of HLA-DR revealed normally distributed resting microglia in both the white and grey matter of two narcoleptic subjects. Neither of the cases (aged 77 and 67 years) was associated with activated, amoeboid microglia. This is remarkable, since the upregulation of HLA-DR expression and microglia activation are hallmarks of immune-mediated inflammation in the CNS. In the context of a description of dysregulated tumour necrosis factor (TNF) expression (see below) it is interesting to note that *in situ* hybridization for TNF RNA did not reveal a significant signal in control and narcoleptic tissue (Peyron *et al.*, 2000). Taken together, these findings do not support the model that T cells and macrophages induce a reduction in the numbers of hypocretin neurons. However, altered MHCI, MHCII and TNF expression may only be seen early on, at the time of the loss of hypocretin neurons, but not years after onset of disease.

Are TNF-α and its receptors critical for the pathogenesis of narcolepsy?

A growing body of evidence supports a role of the cytokine TNF-α in sleep disorders, including narcolepsy, fatigue in infectious and autoimmune diseases and in sleep apnoea. TNF is a homotrimeric cytokine that binds to two receptors, TNF receptor 1 and TNF receptor 2. TNF is synthesized as a type-2 transmembrane protein that is inserted into the membrane as a homotrimer and cleaved by the matrix metalloprotease TNF-α converting enzyme (ADAM 17) to a 51 kDa soluble circulating trimer (Idriss and Naismith, 2000). Both membrane-bound and soluble forms are mainly produced by monocytes, macrophages and dendritic cells. In the context of sleep disorders, it is of note that TNF is produced in the CNS, mainly by microglia cells and astrocytes, but also by neurons (Frei *et al.*, 1989; Lieberman *et al.*, 1989; Probert and Akassoglou, 2001). TNF binds to TNF receptors 1 and 2, which are membrane glycoprotein receptors. TNF receptor 1 is expressed on all types of cells and binds membrane-bound TNF as well as soluble TNF. This contrasts with TNF receptor 2, which is mainly expressed on cells of the immune system, including microglia, and by endothelial cells, and which binds only membrane-bound TNF (Wajant *et al.*, 2003). TNF is a pleiotropic inflammatory cytokine that acts on parenchymal cells in various organs, including the CNS, in which it modulates the functions of microglia, oligodendrocytes, astrocytes and neurons. With respect to sleep disorders it is of note that TNF alters glutaminergic transmission and synaptic plasticity and scaling. TNF increases α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors on neurons and leads to inhibition of the expression of $GABA_A$ receptors, which together leads to increased excitatory synaptic transmission (Campbell and Trowsdale, 1993; Beattie *et al.*, 2002; Stellwagen *et al.*, 2005; Stellwagen and Malenka, 2006). The TNF locus is situated within the Class III region of the human MHC complex on chromosome 6p21. In light of the association of narcolepsy with HLA-DQB1*0602 it is interesting to study the expression of this cytokine in narcoleptic patients.

Interleukin-1β, interleukin-1 receptor antagonist, interleukin-2, TNF and lymphotoxin-alpha in plasma and in mitogen-stimulated monocytes and lymphocytes were not found to differ between narcolepsy patients and HLA-DR2 matched control subjects (Hinze-Selch *et al.*, 1998). Only interleukin-6 was found to be increased in lipopolysaccharide activated monocytes in narcolepsy. However, increased TNF and interleukin-6 serum levels compared to age- and gender-matched controls were detected in a later study by Okun *et al*. (2004), who found the TNF concentration in patients' sera to be 13.9 ± 1.39 pg/ml (control: 8.2 ± 0.45 pg/ml) and the interleukin-6 concentrations to be 6.7 ± 1.45 pg/ml (control: 0.49 ± 0.09 pg/ml for IL-6) (Okun *et al.*, 2004). In the latter study, stimulatory drugs were associated with lower TNF levels. As outlined above, genetic polymorphism in the *Tnf* promoter may also influence TNF serum concentrations. The TNF allele with the C-857T polymorphism was strongly associated in the subgroup of DRB1*15/16 (HLA-DR2 type) negative patients (Wieczorek *et al.*, 2003). In a recent, well controlled study, new information was obtained by Himmerich *et al.* (2006). Whereas serum TNF was not increased, narcoleptic patients had higher soluble TNF receptor 2 (but not soluble TNF receptor 1) compared to controls. This may be explained by genetic polymorphisms. Positive correlations have been observed for the TNF (-857T) and TNF receptor 2 (-196T) combination with narcolepsy, and for DRB1*1501 and TNF (-857T) (Hohjoh *et al.*, 2001*a*, *b*). Further studies should address the relationship between soluble TNF receptor 2 and HLA-DR2.

Collectively, there is evidence that TNF and soluble TNF receptor serum concentrations are abnormal in patients with narcolepsy and that there are *Tnf* and *TNF receptor 2* gene polymorphisms that are linked to the HLA-DQB1*0602 allele. It is of note, however, that the TNF promoter -857T allele, which correlated with the presence of the TNF receptor 2 -196T allele in narcolepsy, has been found to be associated with an almost twice as high TNF produced by blood mononuclear cells (Hohjoh and Tokunaga, 2001*c*). Elevated plasma levels of soluble TNF receptor 2 have not only been detected in narcolepsy, but also in inflammatory diseases including rheumatoid arthritis (Glossop *et al.*, 2005). The extent of production of membrane-bound TNF, soluble TNF and TNF receptor in the hypothalamus at onset of disease has not yet been explored. It is open whether production of TNF and TNF receptor 2 follows activation of MHCII expressing cells and neurons, or follows tissue injury (Knoblach *et al.*, 1999).

The studies on narcolepsy outlined above are also intriguing because subcutaneous infusion of TNF impairs locomotor activity in mice and lowers the expression of clock genes in the liver. TNF acts on clock genes that are regulated by E-boxes in their promoters—namely the PAR bZip clock controlled genes *Dbp*, *Tef* and *Hlf* and the period genes *Per1*, *Per2* and *Per3*—but not *Clock* nor *Bmal1*, which lack E-boxes in their regulatory DNA regions (Cavadini *et al.*, 2007). Since clock genes are central in the sleep-wake cycle and map to mouse chromosome 5 within a region syntenic to human chromosome 4g12, a region close to the narcolepsy susceptibility locus 4p/3-q21 identified recently, polymorphisms have been analysed in the *Clock* gene (Nakayama *et al.*, 2000). However, no differences in allelic and genotypic frequencies of two clock polymorphisms have been observed in narcolepsy compared to controls (Moreira *et al.*, 2005).

Death of hypocretin expressing neurons in narcolepsy from an immunological point of view

Taking into account the immunological features of narcolepsy outlined above (HLA-DQB1*0602 association, polymorphisms in the TNF/TNF receptor genes and in the TCRα locus, anti-Trib2 antibodies), death of hypocretin neurons in narcolepsy could be immune mediated. T cell cytotoxicity mediated by MHCI restricted neuronal killing is unlikely. Antigen-specific lysis of target cells by cytotoxic CD8$^+$ T cells requires expression of MHCI antigens (Walter and Santamaria, 2005). However in the normal CNS, these molecules are not expressed, or only at low levels, in synapses and dendrites of neurons (Goddard *et al.*, 2007). The induction of MHCI molecules and β2-microglobulin also depends on membrane depolarization (Neumann *et al.*, 1995; Rensing-Ehl *et al.*, 1996). In autoimmune and viral diseases of the CNS there is still no convincing evidence for MHCI-dependent killing of neurons by CD8$^+$ T cells. The sensitivity of neurons to cytotoxic CD8$^+$ T cell-mediated killing has only been demonstrated convincingly with neurons transfected with a gene encoding an MHC class I molecule (Joly *et al.*, 1991; Rall *et al.*, 1995).

MHCII molecules such as HLA-DR2 and HLA-DQB1*0602 bind (self) antigens and interact with antigen-specific TCRs on CD4$^+$ T cells (Chen *et al.*, 2009). MHCII in the CNS is only expressed by microglia and by blood derived monocytes, perivascular macrophages and dendritic cells. These cells may activate invading CD4$^+$ T cells in an antigen and MHCII dependent manner. However, autoptic studies in narcolepsy have detected neither T cells nor increased MHCII expression in the hypothalamus. Since these studies were performed with tissues from patients with long lasting disease, it cannot be excluded that the picture might be very different at the onset of narcolepsy. CD4$^+$ T cells may recognize bacterial antigens, e.g. streptococcal antigens and/or self-antigens. The latter could be a consequence of molecular mimicry between host and pathogen as shown for example in post-streptococcal Sydenham chorea (Kirvan *et al.*, 2003). Patients with narcolepsy ($n = 200$), that are characterized by being DQB1*0602 positive and low hypocretin in the CSF, have recently been found to have increased antibodies to streptolysin and to DNAseB within the first 3 years after onset of disease when compared to age-matched controls ($n = 200$) (Aran *et al.*, 2009). C-reactive protein levels were not increased. Antibodies to streptolysin and antibodies to DNAseB titres were highest close to narcolepsy onset, and decreased with disease duration. This contrasts with anti-*Helicobacter pylori* antibodies, which did not differ from controls. Clinical studies showed that the risk of narcolepsy in a person with a history of a physician-diagnosed streptococcal throat infection before age 21 was 5.4-fold higher than in individuals without such a history (Longstreth *et al.*, 2009). Twenty years ago, increased streptolysin and DNAseB antibody titres were observed in a small number of narcoleptic patients, although this finding was not reproduced later (Billiard *et al.*, 1989; Mueller-Eckhardt *et al.*, 1990). Group size and the time interval between onset of disease and blood testing may contribute to differences in the data.

Immune-mediated bystander killing of neurons

In narcolepsy, the loss of neurons is very selective and includes neurons expressing hypocretin-1, but not neighbouring neurons that produce melanin-concentrating hormone (Blouin *et al.*, 2005; Thannickal *et al.*, 2009). Selectivity could be due to neurotoxic autoantibodies binding to unique antigens expressed only by hypocretin neurons. However, such antibodies have not been detected so far (see above).

Because neurons express only low levels of MHCI/II, T lymphocytes are unlikely to interact in an antigen-dependent way with neuronal cells. However, T cell-mediated killing of neurons could be indirect, neurotoxicity being due to T cell-mediated bystander killing. For example, CA1 hippocampal neurons expressing a transgene encoding the nucleoprotein of Borna disease virus showed no damage when mice were injected with Borna disease virus-specific $CD8^+$ T cells (Richter *et al.*, 2009). This contrasts with mice expressing the nucleoprotein of Borna disease virus in astrocytes. In this situation, Borna disease virus-specific $CD8^+$ T cells were found to interact with regional nucleoprotein positive astrocytes and thereby caused collateral damage to uninfected CA1 neurons. This effect was thought to be the result of neurotoxic molecules released from functionally impaired astrocytes (Richter *et al.*, 2009). Using glutamate receptor antagonists, death of uninfected hippocampal CA1 neurons due to glutamate receptor overstimulation was excluded. The authors suggest that the interaction of $CD8^+$ T cells with astrocytes may impair astrocyte-mediated detoxification of potentially neurotoxic molecules or the production of neurotrophic factors by astrocytes (Richter *et al.*, 2009). Likewise, anti-self $CD8^+$ T cells may cause bystander toxicity by acting on astrocytes expressing MHCI, the cellular interaction causing death of hypocretin neurons. Bystander neurotoxicity has also been observed to develop in the course of the generation of cytotoxic $CD8^+$ T cells that recognize virus (e.g. JC virus) infected MHCI positive oligodendrocytes (for review see Melzer *et al.*, 2009). Another example of bystander neurotoxicity is provided by studies using ovalbumin-specific $CD4^+$ T cells. These cells lead to a lethal MHCII- and antigen-independent increase in neuronal calcium, which could be prevented by blocking glutamate receptors and perforin (Nitsch *et al.*, 2004).

Neurotoxicity by $CD4^+$ T cells may either be mediated by their release of neurotoxic molecules, including interferon gamma (IFNγ), TNF and lymphotoxin α or be due to their ability to activate microglia to produce the aforementioned cytokines, as well as radical oxygen intermediates, nitric oxide and glutamate (Fig. 1) (Piani *et al.*, 1992). TNF by itself is capable of inducing largely unselective, high-conductance ion channels following pH-dependent insertion into lipid bilayer or cell membranes (Kagan *et al.*, 1992; Baldwin *et al.*, 1996). It is interesting that in rat hypothalamic slice cultures the addition of N-methyl-D-aspartate resulted in a marked decrease in the number of hypocretin neurons, whereas neurons expressing melanin-concentrating hormone were relatively spared (Katsuki and Akaike, 2004). Quinolinic acid, a tryptophan metabolite produced by the kynurenine pathway, possesses an agonist activity on N-methyl-D-aspartic acid receptors and leads to selective loss of hypocretin neurons. Microglia cells and blood-derived monocytes/macrophages are prominent sources of quinolinic acid (Stone, 2001). Hypocretin neurons express N-methyl-D-aspartic acid receptors, the glutaminergic inputs regulating their electrical activity (Li *et al.*, 2002). In contrast to neurons expressing melanin-concentrating hormone, hypocretin neurons are inhibited by elevation of extracellular glucose, the effect being mediated by two pore potassium (K_{2P}) channels (Burdakov *et al.*, 2006). Differences of melanin-concentrating hormone neurons and hypocretin neurons, which may result in selective killing of hypocretin neurons, may also depend on ectopic expression of death receptors including TNF receptor or Fas, or a high degree of sensitivity towards proapoptotic signals. The latter may be due to the absence of anti-apoptotic intracellular molecules or of neuroprotective factors or their receptors. Regulation of apoptosis is central in the neuroprotection and neurodegeneration (Okouchi *et al.*, 2007).

To assess T cell autoimmunity in narcolepsy, further work should examine (i) whether there is a restricted usage of TCR genes; (ii) whether T cell activation and neurotoxic effects of T cells from patients are observed in co-cultures with immortalized hypothalamic neurons transfected with HLA-DR2 genes; (iii) whether signs of narcolepsy develop in humanized severe combined immunodeficiency mice that express human HLA-DR2 genes and are injected with reactivated $CD4^+$ T cells from narcoleptic patients; and (iv) whether monoclonal antibodies with streptococcal reactivity can induce apoptosis of hypocretin neurons *in vitro*.

Superantigen-induced T cell activation and neurotoxicity

An alternative model is that infectious pathogens in the hypothalamus may express superantigens that bridge the TCR on T cells with MHCII molecules expressed by cells such as microglia. Bacterial or viral superantigens concentrate on the surface of antigen presenting cells by binding to MHCII molecules and then engage and crosslink multiple TCR molecules, resulting in strong TCR signalling, T cell activation and cytokine production. IFN-γ released by superantigen activated T cells may lead to neuronal excitotoxicity by intracellular trans-signalling between the IFN-γ receptor and a Ca^{2+}- permeable neuronal α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate/kainate receptor in the absence of extracellular glutamate (Mizuno *et al.*, 2008). As a hypothesis, the narcolepsy associated polymorphism of the TCRα locus, as well as DQB1*0602 on cells such as microglia, might permit only a limited engagement of the TCR in the superantigen-dependent process, and thereby cause only minimal

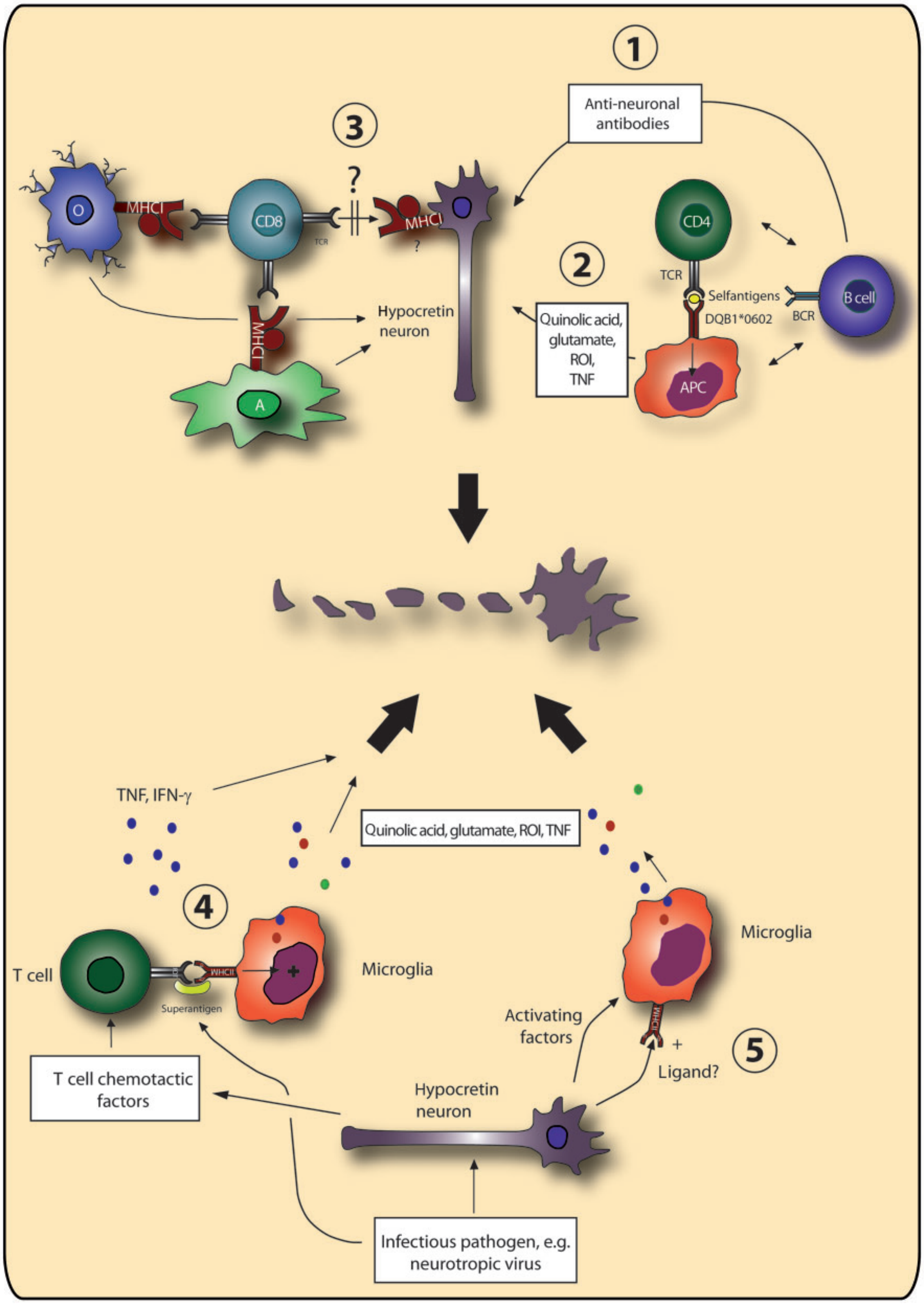

Figure 1 Autoantibody, T lymphocyte and microglia induced killing of hypocretin neurons. (**1**) Hypocretin neurons may be destroyed by autoantibodies such as Trib2 (Cvetkovic-Lopes *et al.*, 2010) which are produced by B cells. (**2**) Priming of $CD4^+$ T cells to self-antigens presented to TCR by DQB1*0602 expressing antigen presenting cells (APC) including dendritic cells, monocytes, macrophages and microglia cells leads to activation of B cells and of antigen presenting cells. The latter produce neurotoxic factors such as quinolinic acid, glutamate, radical oxygen intermediates (ROI) and TNF-α (Katsuki and Akaike, 2004). (**3**) Since the significance of the expression of MHCI

(Continued)

inflammation (Fig. 1). A stronger interaction would be inconsistent with the limited pathology and destruction of only one type of cell—the hypocretin neurons in the hypothalamus. Most superantigens interact with TCR molecules by binding primarily to the variable region of the β chain (for review: Fraser and Proft, 2008). It might thus seem unlikely that the recently observed polymorphism in the TCRα locus could reflect the involvement of superantigens in narcolepsy. However, there are also superantigens that bind to alpha chains of the TCR. The staphylococcal toxin staphylococcal enterotoxin H recognizes the variable region of the TCRα-chain (Vα27) (Pumphrey *et al.*, 2007) and the mycoplasma arthritidis mitogen consists of two α-helical bundles, one of which binds orthogonally to the top of the MHCIIα1-helix, peptide and β1-helix (Zhao *et al.*, 2004).

T cell-independent MHCII-mediated neurotoxicity

In T cell-mediated diseases of the CNS, such as multiple sclerosis or experimental autoimmune encephalomyelitis, the function of MHCII is primarily that of antigen presentation by microglia and macrophages. However, in conditions such as Huntington's disease and Parkinson's disease, or brain trauma, the aforementioned types of cells express MHCII despite the fact that no evidence for T cell involvement has been observed. In these diseases, upregulation of MHCII in microglia cells may follow the ingestion of apoptotic cells (Hellendall and Ting, 1997). Necrotic neurons have been shown to activate microglia to upregulate MHCII, costimulatory molecules CD40, CD24, β2 integrin, CD11b, inducible nitric oxide synthetase and cytokines including TNF (Pais *et al.*, 2008). It has been suggested that an alternative role for MHCII might involve signal transduction leading to activation, differentiation and production of proinflammatory cytokines. Cuprizone-induced oligodendrocyte dysfunction with T cell-independent demyelination pathology is much less pronounced in MHCII $I-A^{-/-}_{\beta(\mathrm{beta})}$mice, or in mice expressing a truncated I-$A_{\beta(\mathrm{beta})}$ chain lacking a cytoplasmic domain. It is not clear yet how signalling by MHCII molecules is activated in the absence of T cell function (Matsushima *et al.*, 1994; Hiremath *et al.*, 2008). These findings may be of relevance for narcolepsy. As a hypothesis, infectious pathogens may have a tropism for hypothalamic hypocretin neurons and thereby cause these neurons to activate microglia to increase signalling via their MHCII molecules (Fig. 1). Activated microglia induce neurotoxicity by releasing quinolinic acid or through the upregulation of glutaminase, an enzyme that produces the N-methyl-D-aspartic acid receptor agonist glutamate (Piani *et al.*, 1991, 1992; Pais *et al.*, 2008). The activation of microglia by necrotic neurons was shown to be dependent on the toll-like receptor-associated adaptor molecule myeloid differentiation primary response gene (*MyD88*).

Conclusion

Autoimmunity, superantigen-mediated T cell activation and non-T cell-mediated activation by MHCII signalling could be involved in narcolepsy. Whereas HLA-DQB1*0602 might select for recognition of self-antigens and thereby lead to autoimmunity, the polymorphism of the TCRα (alpha) gene might be crucial in superantigen-mediated T cell activation. The existence of $CD8^+$-mediated neuronal cell death has not yet been convincingly proven *in vivo*. However, $CD8^+$ induced damage of MHCI expressing astrocytes or oligodendrocytes may be followed by collateral toxicity to neurons. HLA-DQB1*0602 might be more vulnerable to T cell-independent, MHCII-mediated activation of macrophages and microglia. These cells have been shown to release neurotoxic molecules including Fas ligand, proteases, TNF, quinolinic acid, glutamate, radical oxygen intermediates and nitric oxide. Selectivity of killing of hypocretin neurons may be due to a high degree of sensitivity towards the aforementioned cytotoxic molecules as has been shown with quinolinic acid. The detection of an increased frequency of streptococcal infection in narcolepsy may provide a hint for molecular mimicry and autoimmunity or for the involvement of superantigens as initiators of T cell activation. For the detection of anti-CNS antibodies and activation of T cells/monocytes in the blood, future studies should concentrate on patients with a newly diagnosed or very recent onset of disease.

Funding

The Swiss National Science Foundation (Project No.: 310030_125496 to A.F.), the National Centre of Competence in Research Neuro (to A.F.) and the Lotex Stiftung (to A.F.). Adriano Fontana is Hertie Senior Research Professor Neuroscience of the Gemeinnützige Hertie-Stiftung.

Figure 1 Continued
molecules on neurons *in vivo* is still a matter of debate, the contribution of $CD8^+$ T cell-mediated killing of neurons remains open. However there is evidence for $CD8^+$ T cell-mediated antigen-dependent interaction with MHCI expressing astrocytes (A) or oligodendrocytes (O) which as reported may result in damage of these glial cells and collateral toxicity of neurons (Melzer *et al.*, 2009; Richter *et al.*, 2009). (**4**) Neurotoxic molecules may also be released by antigen presenting cells that bind bacterial or viral superantigens via MHCII molecules. Crosslinking of TCRs on T cells leads to release of neurotoxic molecules including interferon gamma (IFN-γ) and TNF. (**5**) Activation of microglia cells or other antigen presenting cells including dendritic cells and monocyte derived macrophages may also be mediated by signalling through MHCII molecules in the absence of T cells (Matsushima *et al.*, 1994; Hiremath *et al.*, 2008). The ligand, which binds to MHCII as well as the origin of the ligand (hypocretin neurons?), are not known. MHCII-mediated activation of antigen presenting cells leads to the release of neurotoxic molecules.

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